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Comparison of the PremiTest and xMAP Salmonella serotyping assays and classical serotyping for determination of Salmonella serovars

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Comparison of the PremiTest and xMAP *Salmonella* serotyping assays and classical serotyping for determination of *Salmonella* serovars

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ABSTRACT

Two commercial molecular assays, the PremiTest *Salmonella* and the xMAP *Salmonella*, were recently developed with the goal of identifying many of the most common *Salmonella enterica* serovars in less than eight hours. This study compared the results of these two assays to that of traditional serotyping on 233 isolates representing more than 52 different serovars, including the ten most frequently identified at the NVSL from 2000 to 2010. The PremiTest assay completely and correctly identified 150 isolates (64%) and the xMAP assay identified 181 isolates (78%). Both molecular assays were able to partially identify additional serovars. These assays offer a new, rapid approach to screening *Salmonella* isolates in the laboratory.

CHAPTER 1. REVIEW OF LITERATURE

Salmonella is a genus of Gram-negative, rod-shaped, non-sporulating bacteria in the family Enterobacteriaceae. The salmonellae are facultative anaerobes that ferment glucose and are able to use citrate as a sole carbon source. They are motile by means of peritrichous flagella, with the exception of the non-motile *Salmonella* serovar Gallinarum [1-4]. The genus consists of two species; *Salmonella enterica* and *Salmonella bongori*. Each subspecies is designated with a Roman numeral as well as a name. *Salmonella bongori* (V) was originally considered a subspecies, so the original Roman numeral designation was kept to avoid confusion. *Salmonella enterica* is further divided into six subspecies; *Salmonella enterica* subspecies *enterica* (I), *Salmonella enterica* subspecies *salamae* (II), *Salmonella enterica* subspecies *arizonae* (IIIa), *Salmonella enterica* subspecies *diarizonae* (IIIb), *Salmonella enterica* subspecies *houtenae* (IV) and *Salmonella enterica* subspecies *indica* (VI) [3, 5]. Speciation is determined by the differences in abilities to utilize various chemical compounds for growth. *Salmonella enterica* subspecies *enterica* serovars are found to cause over 90% of *Salmonella* infections in humans. *Salmonella enterica* subspecies *salamae*, *arizonae* and *diarizonae* are most often found in the intestines of reptiles and other cold blooded animals, although *arizonae* can be problematic in turkey poults and sheep [3]. *Salmonella enterica* subspecies *houtenae* and *Salmonella bongori* are most often found in the environment and are not typically pathogenic to man [6]. *Salmonella subterranean* was formerly considered to be a third species, but was recently determined to not belong to the genus *Salmonella* based on DNA relatedness studies [5]. *Salmonella* is ubiquitous and found

worldwide in water, dust, soil, insects, raw meat, poultry, eggs and seafood. Salmonellae are enteric pathogens of man and animals, particularly livestock and poultry. Various serovars have been found on surfaces of vegetables, feeds and fruits, likely due to fecal contamination [2, 6, 7]. They are able to survive and thrive in a wide variety of conditions, growing at temperatures ranging from 8-45°C, pH of 4-9, and in environments of up to a 20% salt concentration. They are resistant to drying and thought to survive in dust for years [2].

Salmonella serovar Typhi was first described in 1880 by Eberth. The first isolation of what would later be known as *Salmonella* serovar Choleraesuis, was made in 1885 when Theobald Smith, under the direction of Daniel Salmon, isolated what was believed to be the causative agent of hog cholera. They named their organism *Bacillus Cholera-Suis* [3, 4, 8]. In 1900, the genus name of *Salmonella* was officially given by Lignères [4, 6]. The first serological testing of *Salmonella* occurred in 1896 when Gruber and Durham demonstrated the agglutination of typhoid bacterial cells by serum collected from patients with typhoid and from animals that had been inoculated with the typhoid bacteria [4, 9]. In 1903, Smith and Reagh described the differing reactions of the antigens of *Salmonella*, but their work was largely ignored at the time [1]. In 1918, Felix and Weil described the O and H antigens and Andrews found that *Salmonella* had two distinct “H” phases in 1922 [4]. White developed an antigenic scheme for typing of *Salmonella* in 1926, and Kauffmann published a scheme in 1929 [1, 4]. In 1934, the scheme was updated, and the first official Kauffmann-White Schema was published. The World Health Organization Collaborating Centre for Reference and Research on *Salmonella*

(WHOCC-Salm) is responsible for maintaining the list of serovars, the validation of newly discovered serovars and for continuous update of the scheme containing more than 2500 serovars. This scheme is considered the “gold standard” for typing of *Salmonella* and is used throughout the world [1, 3, 5, 6]. The Kaufmann-White scheme describes the antigenic formula that makes up a particular *Salmonella* serovar. In the case of *Salmonella enterica* subspecies *enterica*, a name is also given. The serovar is comprised of a Roman numeral to indicate the subspecies; the somatic (O) antigen; the first flagellar (H1 antigen) and the second flagellar (H2) antigen, if present. The O, H1 and H2 antigens are each separated by a colon [5].

Salmonellosis is considered to be the most frequently occurring foodborne illness worldwide [2, 6, 8]. Illness from ingestion can present as enteritis, paratyphoid fever or typhoid fever. The human health impact and financial implications from infection with *Salmonella* are staggering. This burden is felt in both developed and non-developed countries. Developed countries tend to have high numbers of gastroenteritis infection due to the nature of commercially prepared food and food products [9, 10]. It has been estimated that there are between 2 to 4 million people that are infected with non-typhoidal salmonellosis in the United States annually, with between 500-1000 deaths resulting from complications of infection. The financial burden associated with *Salmonella* in the United States alone is estimated to exceed 3 billion dollars annually [9, 11]. Developing countries are more likely to experience the problems of typhoid and paratyphoid fevers due to issues of contaminated water sources [7]. World Health Organization (WHO) numbers estimate that there are approximately 16.6 million cases of

typhoid annually, and over 600,000 people die each year worldwide from *Salmonella* serovar Typhi [9]. Infectious dose can be as low as one cell and as high as 1 x 10^{12} cells, depending on the virulence attributes of the particular organism as well as the susceptibility of the host [2, 7, 12]. Some serovars of *Salmonella* are host adapted: *Salmonella enterica* serovar Dublin in cattle, *Salmonella enterica* serovar Gallinarum in poultry, and *Salmonella enterica* serovar Choleraesuis in swine are a few examples. Although these serovars are considered to be host adapted, all *Salmonella* can be pathogenic in man, and when these serovars cause infection in man, the disease tends to be severe and systemic. They often cause higher mortality rates, as they tend to occur in the very young, the elderly and the immune compromised. Even very low numbers can cause significant disease in individuals who are more susceptible to infection. Infants tend to be very sensitive to *Salmonella* infections and can experience severe complications such as mucopurulent bloody diarrhea leading to severe dehydration, toxicosis and meningitis [7, 13]. For the average individual, gastroenteritis symptoms begin within 6-72 hours after consumption of food containing a large number of organisms. Symptoms include abdominal cramping, nausea with occasional vomiting, diarrhea, fever, and headache. The disease is usually self-limiting, and lasts from 2-7 days. Administration of antibiotics frequently prolongs shedding [2, 5, 7, 14]. Symptoms of typhoidal salmonellosis appear 1-4 weeks following ingestion of *Salmonella* serovar Typhi or Paratyphi organisms and include high fever, diarrhea or constipation, appearance of classical “rose spot” rash and extreme fatigue. If untreated, typhoid fever can have mortality rates as high as 10 to 15%. Disease usually lasts 2-4 weeks, although

an individual can continue shedding the organism for months and occasionally years [2, 7, 8].

Salmonella is also a problem in animals. As is the case in humans, infections caused by *Salmonella* in animals can present at a subclinical level, where no signs of illness are present, but the animal maintains the infection and intermittently or continuously sheds the organism into its environment. Animals may exhibit signs of mild to severe enteritis and occasionally, a typhoid-like illness capable of causing systemic infection and death. Severe illness is most often associated with species-specific *Salmonella* serovars that occur in neonates or adult animals that are stressed from deprivation of food and water, poor nutrition, crowded conditions, periods of long transportation, illness and injury, or pregnancy. Often, infection with the same serovars in normal healthy adult animals tends to cause a mild to sub-clinical infection, but leaves the animal in a carrier state [15-19]. In poultry, *Salmonella* serovar Gallinarum var. Pullorum is associated with significant disease in young chicks. Many die prior to or just after hatching. Chicks that hatch exhibit a characteristic white diarrhea with pasting of the vent and ruffling of the feathers. Mortality is typically very high. Another biovar, *Salmonella* Gallinarum var. Gallinarum, is the causative agent of fowl typhoid. Fowl typhoid causes illness in all ages of poultry. Symptoms can include yellow diarrhea with mucus, depression, respiratory distress, decreased egg production and sporadic death [16, 19-21]. *Salmonella enterica* subspecies *arizonae* can cause significant problems in turkey poults less than 3 weeks of age with mortality rates of up to 80%. Symptoms include diarrhea, pasting of the vent, anorexia, listlessness, ruffled feathers and shivering. Those that do survive typically become long

term carriers. Adults typically are asymptomatic although a decrease in egg production can occur and they tend to shed the organism for long periods of time [15, 22]. *Salmonella* serovar Typhimurium and *Salmonella* serovar Dublin both cause clinical disease in calves, however, *Salmonella* serovar Typhimurium tends to cause significant illness in calves less than one month old, whereas *Salmonella* serovar Dublin becomes problematic in calves that are between two and three months old. Symptoms include watery diarrhea with mucus or blood present, fever, dehydration, sepsis, pneumonia, and moist cough. Losses can be very high, and death can occur shortly after signs develop. Adult cattle occasionally develop mild enteritis, pregnant cows may abort, and milk production can decrease. Adults tend to shed *Salmonella* for months to years, especially in times of stress [15, 23-25]. *Salmonella* infections in adult swine are not common, and typically can be traced to the purchase of an infected animal. *Salmonella* serovar Typhimurium is known to cause diarrhea, anorexia, and fever in young piglets, although mortality is not high. *Salmonella* serovar Choleraesuis can infect swine of all ages. Swine present with fever, anorexia, depression and respiratory distress leading to cyanosis; mortality from infection can be quite high [15, 26]. *Salmonella* infections can cause problems in equines of all ages. *Salmonella* serovar Abortusequi is a causative agent of abortion in pregnant mares and was first described in 1893. Other *Salmonella* serovars can persist in the mare without detection throughout pregnancy, and the stress of foaling can cause sudden shedding with the foal being infected during or immediately after birth. *Salmonella* infection in foals tends to be systemic, with signs of depression, fever, and severe diarrhea leading to dehydration and electrolyte imbalance. Endocarditis, pneumonia, meningitis and infection of the joints can also occur. Mortality can be high.

Salmonella is also a significant issue in hospitalized horses of all ages [15, 18, 27]. Typically, *Salmonella* infections in dogs and cats are uncommon, however, there have been infections that have been traced back to dry pet food in recent years [28]. Younger puppies and kittens are more likely to become ill from *Salmonella*, and mortality is very low, although carrier status may last for several weeks. Typical signs are gastroenteritis, fever and lethargy [15, 29].

The ability of *Salmonella* to survive in many different environments and produce a carrier state in relatively healthy humans and animals presents a significant challenge for the control of infection. This contributes significantly to the problem of foodborne disease on many levels. Ideal control includes removal of diseased animals from the food chain; however, it is not easy to identify carrier animals when they appear asymptomatic. As apparently healthy carriers leave the farm for slaughter, the conditions of crowding, withholding of feed and transport cause stress to the animal. As they are being transported, the stress of this leads to the shedding of *Salmonella* into the environment from animals that have shown no visible sign of infection. The close proximity allows previously unexposed animals to become infected or physically contaminated.

Meat and poultry inspection processes work to protect consumers by providing pre- and post-mortem inspection of animals, prevention of diseased meat from entering into the food chain, continuous inspection of products through processing, and setting guidelines for strict hygiene within the processing plant environment to prevent contamination. The United States first passed federal legislation on meat inspection in 1890 which was followed by the Meat Inspection Act of 1906. This was regulated by the USDA’s Animal

Inspection Agency under Dr. Daniel Salmon. The Poultry Products Inspection Act passed by Congress in 1957 provided similar guidelines for the inspection of poultry and poultry products. Many of the standards of these acts are still in use today [30, 31].

Surveillance and inspection programs help to track the occurrence of *Salmonella*, as well as the changes that occur over time with the goal of identifying potential hazards or critical points in which *Salmonella* contamination can enter the farm-to-fork chain of food production. Programs such as Foodborne Diseases Active Surveillance Network (FoodNet), which include both public health organizations such as the Centers for Disease Control (CDC), state health laboratories, and the Food and Drug Administration (FDA) as well as veterinary organizations like the United States Department of Agriculture's (USDA)-Food Safety Inspection Services (FSIS) and the USDA-Animal and Plant Health Inspection Services (APHIS), collaborate to actively track laboratory confirmed cases of *Salmonella*. It is critical that both human health and veterinary diagnostic laboratories work together, as animals are the principal vectors of zoonotic salmonellosis [7, 32, 33]. Any animal or animal product can potentially be infected with or become contaminated with *Salmonella*. Some serovars are able to better survive and thrive than others, and although most serovars have the potential to cause disease in man, five to ten account for the majority of cases of salmonellosis that are seen in both man and animals [34, 35]. *Salmonella* serovar Enteritidis has been linked to many human health outbreaks and most often is associated with poultry and eggs. Hens are often infected without any visible sign, and can be laying eggs that harbor the bacterium. *Salmonella* serovar Enteritidis infections in humans can often be traced back to

contaminated shell eggs that are served ‘sunny-side-up”, soft boiled or lightly scrambled or have been used in foods that are not cooked or are undercooked [2, 7, 16, 19, 36]. Programs such as the USDA’s National Poultry Improvement Plan (NPIP) and the FDA’s shell egg program were established to decrease the prevalence of *Salmonella* in both poultry and in shell eggs on the farm through regulations that include implementation of biosecurity programs, incorporation of measures to control insects, rodents and wild bird access, hygienic disposal of animal wastes, ensuring that feed and water are free from *Salmonella*, proper disinfection procedures, monitoring of the environment and testing of animals and eggs for the presence of *Salmonella*. These agencies also define rules for diversion of eggs and culling of flocks as well as disinfection procedures if monitoring samples show that *Salmonella* serovar Enteritidis is present [19, 36, 37].

Salmonella serovars Typhimurium, Enteritidis, Heidelberg, Montevideo, Newport, Javiana and 4,(5),12:i:- were recognized as serovars that cause up to 64% of illness in man. These serovars are most often found on carcasses of chicken, turkey, cattle and swine, and in ground beef, ground turkey and ground chicken [12, 19, 22, 25, 26, 34, 38]. FSIS implemented the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems in 1996 which established performance standards for the prevalence of *Salmonella* in facilities that slaughter animals or those that produce raw meats or raw ground products. These standards are based on the number of samples per set that are positive for *Salmonella*, as opposed to the number of organisms that are present. The number of samples and the number of positives allowed for each set are based on the class of product. Updates to the rule were made in 2006 and 2008 which focus on the

prevalence of serovars of *Salmonella* that are of human health concern as part of the surveillance process. When considering the number of positive samples that are considered acceptable, only a percentage of those can be of serovars of public health significance. Facilities that are not able to maintain consistently low numbers of *Salmonella* and serovars that are most likely to cause foodborne illness in man are subject to additional surveillance by FSIS [12, 38].

Identification of *Salmonella* serovars in animals, their environment, meat, eggs and other food products provides critical information for surveillance, status of a herd, the successes of manufacturing processers to control the spread of pathogens, and epidemiology in the event of foodborne illnesses. For more than 70 years, classical serotyping of *Salmonella* has been performed using standardized animal antisera to test for the lipopolysaccharide (O antigen) and the flagellar proteins (H antigens) in accordance with the methods described by Kauffmann and White. The scheme, which was first published in 1934, is still utilized throughout the world and contains over 2500 different serovars [1, 3, 8]. Although classical serotyping is considered to be the "gold standard" of testing, there are several disadvantages. Because classical serotyping is dependent upon phenotypic expression of the O and H antigens, those isolates that express a rough O antigen or have lost the ability to express one or both flagellar antigens are not able to be completely typed. Fimbriated and encapsulated strains also mask the phenotypic expression of the O antigens [3]. Laboratories that wish to test for the 2500 plus serovars of *Salmonella* are required to maintain more than 160 different antisera. Commercial antisera are available for the most commonly typed *Salmonella* antigens,

however, the sera can be expensive and extensive quality control with a wide variety of *Salmonella* serovars must be performed to assure that the sera are reacting as expected. Several additional antisera are not commercially available, which limits the abilities of many laboratories to the testing of only the most common serovars of *Salmonella*. Complete characterization of isolates for which sera are not available requires that they must be forwarded to a reference laboratory that is able to produce additional antisera in-house. Production of standardized antisera in laboratory animals and the subsequent absorptions take time and are costly to produce. Only a small number of laboratories have these capabilities. Classical serotyping must be performed by staff who are adequately trained and the time required to perform testing can take several days to more than a week [3, 8, 39, 40]. Rapid identification is essential when surveillance on the farm indicates that a serovar such as *Salmonella* serovar Enteritidis may be present so that animals or animal products containing serovars of consequence to public health are identified and excluded from the food chain. Compliance with regulations put forth by FSIS require that results are provided in a timely manner to assist in the identification of contaminated products [36-38]. In order to meet the needs of these regulations, several assays have been developed with the goal of obtaining rapid identification of *Salmonella* serovars of human and veterinary significance. Alternative approaches for replacing classical serotyping include pulsed field gel electrophoresis (PFGE), antibody based microarrays, Multilocus Variable number of tandem repeats Analysis (MLVA), Multilocus Sequence Typing (MLST), PCR, real-time PCR, and DNA sequencing [8, 20, 21, 39-44]. PFGE is considered the “gold-standard” of molecular finger-printing for epidemiological studies, as it is able to identify genomic differences among strains of the

same serovar. However, not all serovars of *Salmonella* show significant genetic diversity on PFGE, including *Salmonella* serovar Enteritidis. Other serovars show significant diversity from isolate to isolate. PFGE requires expensive specialized software to analyze results as well as an extensive database containing the patterns of many isolates. Few laboratories are able to maintain such databases. When a pattern is found that does not match those in the database, additional testing is required. PFGE is also very labor intensive, and less than 15 isolates can be analyzed on a standard gel [8, 45]. An antibody based microarray that is able to identify 20 common *Salmonella* serovars has been described. While the assay is able to test for multiple *Salmonella* antigens at the same time, thus decreasing testing time, it also requires the use of commercially available antisera and is dependent upon the phenotypic expression of the surface antigens of *Salmonella* [46]. Many PCR and Real-Time PCR's have been developed for the rapid identification of *Salmonella* serovars; however, they are typically developed to target one to a handful of serovars. These assays can be useful for laboratories that are performing testing for specific serovars in surveillance programs, such as *Salmonella* serovar Enteritidis in shell egg production. MLST aims to identify genetic relationships within and between serovars through automated DNA sequencing of internal fragments of multiple housekeeping genes. Current assays have only considered a few different serovars of *Salmonella*. Like PFGE, databases to compare results are necessary, and the cost of DNA sequencing will exclude most laboratories from the technology [8, 47, 48]. The ideal assay to incorporate into a laboratory that is performing serotyping will be easy to implement while providing a rapid, accurate result. To be cost effective, it must cover a broad range of serovars, be able to test multiple isolates at one time and the cost of the

equipment and consumables must be reasonable. The results should be easily defined and consistent with the Kauffmann-White scheme, as that is the "gold-standard" of *Salmonella* serotyping. The PremiTest *Salmonella* (PTS) is a commercially available multiplexed DNA assay that was designed to type the most common serovars of *Salmonella*. The PTS selects for genetic markers that are designed to provide unique hybridization profiles for common *Salmonella enterica* subspecies enterica serovars through a process known as multiplex ligation detection reaction (LDR). Typing is based on a set of 14 LDR probes which target genetic markers that differ in the various serovars of *Salmonella*. Each LDR probe contains a unique "ZIP-code." The LDR probes generate circular fragments of DNA from an isolate, which are then amplified through PCR. The amplified products are biotinylated during PCR. Following PCR, the amplified probes are then hybridized onto a low density microarray tube embedded with 100 complementary "cZIP-code" oligonucleotides. Conjugation of biotinylated products is performed with streptavidin-horseradish peroxidase, the conjugate is rinsed away, and a peroxidase substrate is added to provide staining of bound products. Microarray tubes are analyzed with a photometric detector using proprietary software. Because there are multiple steps to the assay, controls have been built into the array tube to indicate success of each step: probe ligation specificity and efficiency, PCR amplification, efficiency of hybridization, detection of the label and the quality of the label. If results indicated assay failure, the controls provide information indicating where the failure occurred. Only the failed steps need to be repeated. The microarray tube is designed to allow three separate isolates to be analyzed at a time.

The PTS assay was tested in parallel with classical serotyping on 754 isolates representing 58 serovars of animal origin from Belgium and the Netherlands in 2007 by Wattiau et al. as directed by the manufacturer on the ATR-03 using CheckPoints software version 4.2 [43]. The DNA was extracted for the subsequent reactions using the provided reagents according to the procedure outlined by the manufacturer. Correct identifications were obtained for 658 isolates (87%), 23 samples (3%) had assay failures, 50 isolates (7%) identified unique genovar codes, 16 isolates (2%) gave dual results, and 7 isolates (1%) gave incorrect identifications. Classical serotyping identified 685 isolates (91%) completely. The remaining 69 isolates (9%) were not completely typed because of rough O antigen or lack of expression of one or both H antigens. The authors found that when assay failures were observed, they most commonly occurred with three serovars; *Salmonella* serovar Typhimurium, *Salmonella* serovar Infantis and *Salmonella* serovar Paratyphi B var. Java. Seven identification errors were observed: *Salmonella* serovar Anatum was incorrectly identified as *Salmonella* serovar Newport, *Salmonella* serovar Borreze was misidentified as *Salmonella* serovar Agona, *Salmonella* serovar Regent was incorrectly identified as *Salmonella* serovar Altona or *Salmonella* serovar Agona, *Salmonella* serovar SanDiego was incorrectly named *Salmonella* serovar Muenster, *Salmonella* serovar Virchow was misidentified as *Salmonella* serovar Minnesota, and two isolates of *Salmonella* serovar Weltevreden were incorrectly typed as *Salmonella* serovar Brandenburg. Another isolate that was incomplete due to lack of flagellar antigen expression on classical serotyping had the antigenic formula of 9,46:-:-. The PTS identified this isolate as *Salmonella* serovar Enteritidis. *Salmonella* serovar Enteritidis does not express O factor 46. Assuming that classical typing of the O antigen is correct,

this isolate would also be incorrectly identified on the PremiTest assay [3, 5]. The authors of this study chose to re-analyze problematic isolates using a purified DNA extract in place of the crude bacterial extract that was described in the assay protocol. Correct identification of 714 isolates (94.4%) was achieved with the use of this DNA for testing. All of the results from assay failure were resolved. The error rate decreased as well: one isolate of *Salmonella* serovar Anatum that had been misidentified as *Salmonella* serovar Newport, was correctly identified on retesting and the isolate of *Salmonella* serovar SanDiego which had been incorrectly identified as *Salmonella* serovar Muenster gave a dual result of *Salmonella* serovar Muenster or *Salmonella* serovar SanDiego when further analyzed using a purified DNA extract. The other five serovars (0.6%) that were described above still gave incorrect results with the second DNA extract.

Meneses also compared the PremiTest assay with classical serotyping. One-hundred reference isolates and 100 isolates that were obtained from poultry and swine operations during the study were tested. Of the 200 isolates tested, 168 had serovars that were included in the PTS database. Of those, 105 isolates (63%) were correctly identified and comparable to the results observed from classical serotyping, 45 (27%) were inconclusive due to assay error, dual results or unique genovar identifications, and 18 (10%) had incorrect serovar names assigned. *Salmonella* serovar Agona and *Salmonella* serovar Collindale were both incorrectly identified as *Salmonella* serovar Montevideo, another isolate of *Salmonella* serovar Agona was misidentified as *Salmonella* serovar Altona, two isolates of *Salmonella* serovar Branderup were incorrectly identified as *Salmonella* serovar Manhattan, *Salmonella* serovar Derby was incorrectly designated *Salmonella*

serovar Adelaide, two isolates of *Salmonella* serovar Enteritidis were also incorrectly identified, one as *Salmonella* serovar Hadar and the other as *Salmonella* serovar Heidelberg. *Salmonella* serovar Fresno was misidentified as *Salmonella* serovar Ouakam or *Salmonella* serovar Meleagridis. *Salmonella* serovar Litchfield and *Salmonella* serovar Tennessee were also incorrectly identified as *Salmonella* serovar Ouakam. An isolate of *Salmonella* serovar Infantis was incorrectly named *Salmonella* serovar Heidelberg, *Salmonella* serovar Kentucky was incorrectly identified as *Salmonella* serovar Ohio, *Salmonella* serovar Livingstone was misidentified as *Salmonella* serovar Lille, *Salmonella* serovar Montevideo was incorrectly named *Salmonella* serovar Schwarzengrund or *Salmonella* serovar Grumpensis, *Salmonella* serovar Muenchen was incorrectly identified as *Salmonella* serovar Newport, *Salmonella* serovar Oranienburg was incorrectly identified as *Salmonella* serovar Monschaui, *Salmonella* serovar Stanley was misidentified as *Salmonella* Muenchen, and *Salmonella* serovar Worthington was incorrectly named *Salmonella* serovar SanDiego. Thirty-two isolates were not included in the PTS database and were not expected to give serovar results; however, 13 isolates were incorrectly identified. *Salmonella* serovar Alachaua was incorrectly identified as *Salmonella* serovar Cubana, *Salmonella* serovar Cape was incorrectly identified as *Salmonella* serovar Thompson, *Salmonella* serovar Essen was misidentified as *Salmonella* serovar Derby, *Salmonella* serovar Fresno was incorrectly identified as *Salmonella* serovar Ouakam or *Salmonella* serovar Meleagridis, *Salmonella* serovar Gaminara was misidentified as *Salmonella* serovar Typhimurium, *Salmonella* serovar Menston was incorrectly identified as *Salmonella* serovar Oranienburg, *Salmonella* serovar Remo was incorrectly named *Salmonella* serovar Schwarzengrund or *Salmonella*

serovar Grumpensis, *Salmonella* serovar Thomasville was incorrectly identified as *Salmonella* serovar Orion, *Salmonella* serovar Try was incorrectly identified as *Salmonella* serovar Typhimurium, two isolates of *Salmonella* serovar Johannesburg were misidentified as *Salmonella* serovar Urbana, and *Salmonella* serovar Menhaden and *Salmonella* serovar New Brunswick were both incorrectly identified as *Salmonella* serovar Give [33].

The Luminex Multianalyte Profiling *Salmonella* serotyping assay (xMAP-SSA) is a multiplexed, nucleic acid-based array that was designed to serotype *Salmonella* isolates based on the genetic markers that determine the O and H antigens. Following a simple DNA extraction, individual PCR reactions are performed for the O and H antigens. The O antigen assay primers are designed to amplify specific genes in the *rfb* region which are responsible for the genetic variations among the individual serogroups. Specific primer and probe sets were designed for each of the serogroups O13, B, C1, C2, D, and E and for *Salmonella* serovar Paratyphi A. The forward primers for the O antigen assay were biotin labeled at the 5' end. The H antigen assay contains nine forward primers and 11 reverse primers that are designed to amplify the variable regions of the *fliC* (H1) and *fljB* (H2) genes. The 5' ends of the reverse primers were biotin labeled for the H antigen assay. The O and H antigen oligonucleotide probes were covalently linked to individual microspheres that were internally dyed with varying concentrations of red and infrared fluorophores. The specific concentrations of the dyes give each bead a "color" that is then associated with a unique spectral address. Each assay consists of a panel of O or H antigen-specific probes linked to one of 100 differently "colored" beads. Following

amplification, the biotinylated PCR products are transferred to a 96-well plate containing the labeled O or H bead sets for hybridization. After binding to the probe-labeled beads, the reporter dye, Streptavidin-Phycoerythrin (SAPE) is added to the reaction. The 96-well plate is placed into the BioRad LX200 for sampling and data acquisition. A sample is drawn up from each well through a flow cytrometry-based system, and the linked microspheres pass through the detection chamber. Each bead is individually analyzed by two lasers. The first laser excites the fluorescent dye of the microsphere and classifies it according to its unique spectral address. The reporter laser analyses the signal and reporter dye content from beads to detect the presence of covalently bound probes that are hybridized to biotinylated PCR products. The results are reported as median fluorescence intensity (MFI) which is automatically calculated in the LX200 software. A positive signal is defined as an MFI giving 6 times the background signal [39, 40].

Fitzgerald et al. described the development and validation of the O antigen assay [40]. The assay was tested on a panel of 393 isolates of *Salmonella* that were obtained from the culture collection of the *Salmonella* Reference Laboratories at the Centers for Disease Control and Prevention. Results were compared to classical typing. Classical serotyping was able to type the O group for 384 isolates (98%). Nine isolates produced a rough O result with classical serotyping. Molecular typing was able to identify 368 of the 393 isolates (94%). Six of the isolates that were considered rough by classical serotyping were successfully typed on the xMAP *Salmonella* assay. Seventeen isolates (4.4%) produced results that were different from classical serotyping but correlated to known genetic relationships between O antigens that are not observed with classical serotyping.

Five isolates (1.3%) did not react with probes that were covered in the assay: two isolates of *Salmonella bongori* (V) and three isolates of *Salmonella enterica* subspecies *diarizonae* (IIIb) did not react with O13.

McQuiston et al. described the development and validation of the H antigen assay [39]. The assay was evaluated on a panel of 500 isolates of *Salmonella* obtained from the culture collection of the *Salmonella* Reference Laboratory at the Centers for Disease Control and Prevention. Testing was performed in parallel with classical serotyping. The H antigen assay was able to completely type 402 of the 500 isolates (80%). Forty-six of the 500 isolates (9%) were partially serotyped, but contained an H1 or H2 antigen that was not in the assay. The H antigen assay was able to correctly type 13 isolates (3%) that were untypeable by classical serotyping because of lack of expression of one or both H antigens. Thirty-nine isolates (8%) did not react with probes that were covered in the assay. Twenty-five of the 39 isolates did not react to the H:5 or H:t probes. It is known that there are multiple genetic lineages for both single factor 5 and single factor t. The bead for the single factor t in the assay represents one of three possible lineages. The bead for the single factor 5 represents most, but not all, lineages. Six isolates of *Salmonella enterica* subspecies *diarizonae* (IIIb) did not react with the H:z probe, one subspecies IIIb isolate did not react with the H:z35 probe. One isolate of *Salmonella enterica* subspecies *salamae* (II) did not react with the H:5 or H:7 probes, and one subspecies II isolate did not react with the H:x or H:z15 probes. Two isolates of *Salmonella* serovar Pensacola did not react with the H:m;m,t probe, two isolates of

9,12:l,v:- did not react with the H:v probe and one isolate of *Salmonella* serovar Putten did not react with the H:d probe. There were no incorrect results.

CHAPTER 2. INTRODUCTION

Salmonella is one of the leading causes of food-borne illnesses accounting for an estimated 93.8 million cases and 155,000 deaths worldwide annually [6]. As infections increase, serotyping continues to be used as the definitive method of identification of the etiological agent and epidemiology. The genus *Salmonella* is comprised of two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* consists of six subspecies and can be further differentiated into more than 2500 serotypes. *Salmonella* serotypes are determined by specific patterns of reactions of their surface antigens with specific *Salmonella* antisera.

Classical antiserum based serotyping using the Kaufmann-White scheme has been the standard method of serotyping *Salmonella* for epidemiological and monitoring purposes for more than seventy years. Classical serotyping relies on the phenotypic expression of the somatic lipopolysaccharide (O antigen) and the flagellar proteins (H antigens) of salmonellae. However, serological testing has several disadvantages, such as the use of more than 160 different antisera to test for the 2,500 plus serotypes of *Salmonella*. While standardized antisera are available for common antigens, there are many others that are not commercially available. Thus, most reference laboratories are required to produce significant volumes of in-house reagents in order to offer complete typing of *Salmonella*. Production of standardized antisera in laboratory animals and the subsequent absorptions take time and are costly to produce. Additional issues that are commonly encountered during serotyping of *Salmonella* include: rough colony variants, leading to an indistinguishable O antigen; isolates that have lost the ability to express one or more

flagellar antigens; fimbriated strains; encapsulated strains; and isolates that express atypical biochemical reactions. The objectivity of the test requires that staff be extensively trained to assure accurate and reproducible results. Finally, the time required to test for complete serovar identification can take several days to more than a week.

From 2000 to 2010, the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL) *Salmonella* Reference Laboratory analyzed 217,627 isolates submitted for serotyping. Twenty serovars accounted for 74% of all isolates that were serotyped at the NVSL during this interval. Typing 18,000-20,000 isolates per year requires a significant amount of time. As surveillance, monitoring, and control programs increase in the agriculture and food industries, the necessity for a faster, reliable test to identify the serovars of public health significance has become paramount.

The objective of this study was to evaluate the potential of high-throughput, real-time molecular typing to replace or augment classical typing at the NVSL. Whereas classical serotyping relies on the phenotypic expression of surface antigens, DNA-based assays utilize stable genetic signatures. Several molecular assays have been developed and used for specific monitoring and surveillance programs; however, the vast majority of those tests can identify only a few serotypes. When considering the molecular assays currently available, this study looked at tests that would identify as many serovars as possible while maintaining the sensitivity and specificity of classical serotyping. Cost, time and technical skills necessary to perform the tests and interpret the results were also considered.

The PremiTest *Salmonella* (PTS) and the Luminex Multianalyte Profiling *Salmonella* serotyping assays (xMAP-SSA) represent two commercially available formats. The PTS is designed for rapid molecular serotyping by the recognition of highly specific DNA targets [43, 49]. A series of ligated probes are generated from genomic DNA, amplified by a polymerase chain reaction (PCR), and hybridized to a DNA microarray with specific primers, one of which is biotin labeled. Amplified biotinylated products are then detected on a photometric detector and analyzed using the proprietary software. Individual serovars are assigned a unique zip code based on a pattern of hybridizations that are produced in the micro-array tube. The assay was initially validated with 443 veterinary strains from Belgium and The Netherlands. A second study evaluated an additional 754 veterinary strains from Belgium [43, 49].

The xMAP *Salmonella* serotyping assay is a system for determination of a serovar based on DNA markers that encode for specific O and H antigens. By using these markers, the xMAP assay amplifies target genes encoding the same antigens that the Kaufmann-White scheme utilizes, thus allowing for direct comparison of the results of a high-volume assay to that of classical serotyping. This provides the unique ability to tie partial results obtained by the assay back into classical testing to complete the serotyping when a particular antigen is not available in the test. The O assay was validated on a panel of 393 strains and the H assay was validated on a panel of 500 strains [39, 40].

Ultimately, the PTS and the xMAP *Salmonella* assays were chosen for this study based on each manufacturer's data which suggested that each assay identified more than 50 different serotypes.

CHAPTER 3. MATERIALS AND METHODS

Isolates. All isolates were obtained from the reference collection at the NVSL. Isolates were originally submitted to the *Salmonella* Reference Laboratory for identification or confirmation of serovar by state or private laboratories where the isolates were recovered from various animal and environmental sources. A minimum of 5 isolates for each of the top 20 most commonly identified serotypes from all animal sources received at the NVSL for the time period of 2000-2010 were chosen for testing along with other serovars of significance to the animal industry. When multiple strains of the same serotype were selected, isolates from different geographical regions and different animal species were chosen, if possible, to reduce the likelihood of testing clonal strains.

In all, 233 isolates representing 52 serovars, 11 isolates not expressing one or more flagellar antigens, 1 isolate that contained multiple serotypes and 7 rough isolates were analyzed (Tables 1 and 2).

Isolates chosen for testing on the xMAP *Salmonella* assay and the PremiTest *Salmonella* assay were assigned unique numbers and tested without the knowledge of their prior identification by classical serological testing.

Classical serological testing. Isolates were serotyped utilizing the method described by Edwards and Ewing [3]. Commercial (Becton, Dickenson and Company, Sparks, Maryland; Remel, Dartford, England; Staten Serum Institute, Copenhagen, Denmark) and in-house prepared antisera were used for typing, as described [3]. Briefly, somatic antigen was prepared by washing the bacterial growth (16-20 hours at 37°C) off of the

bottom half of a blood agar slant with 1mL of 0.85% saline. Ten microliters of antigen and 10µL of antisera were mixed together on a glass slide and gently rocked for 1-2 minutes after which agglutination was scored. All antigens were tested against a minimum of two antisera; most were tested against O groups B, C1, C2, D and E. Following determination of the somatic group, individual factors were tested as appropriate.

To produce the flagellar antigen, a tube containing 5mL of trypticase soy broth with tryptose was inoculated with an isolate and incubated (16-20 hours at 37°C) after which approximately 20mL of 0.85% saline containing 0.6% formalin was added. The antigen was allowed to sit for a minimum of one hour at room temperature following formalization. One milliliter of formalinized antigen was added to a 13x100 test tube containing 25µL of antisera and incubated (50°C water bath for 30 to 60 minutes). In instances where a complex antigen was detected, the individual components of the complex were then tested using 0.5mL antigen against 25µL of corresponding antisera and incubated, as previously described. Serovars were designated according to the antigenic composition listed in the Kauffmann-White Scheme based on the reactivity to individual antisera [3, 5].

PremiTest *Salmonella* assay. The PremiTest *Salmonella* kit was obtained from Check-Points (Wageningen, The Netherlands). All of the required components and reagents for the test were included in the kit. Procedures were followed strictly according to included protocol from the manufacturer. For DNA extraction, isolates were streaked onto nutrient agar plates and incubated (37°C for 16-20 hours). A single isolated colony was sampled

by inserting a toothpick directly into a colony, inoculating it into 100 µL lysis buffer and heating (15 minutes at 400 rpm at 99°C) in a Thermo-Mixer (Eppendorf, Hamburg, Germany). For PCR amplification, 10 µL of DNA extract was added to a strip tube containing 2.5 µL thawed dye solution and 5 µL Solution A. The sample was heated (95°C for 3 minutes), followed by 24 cycles each of denaturation (30 seconds at 95°C), primer extension (5 minutes at 65°C), and a final step of 2 minutes at 98°C in a C1000 Thermocycler (BioRad, Hercules, CA). Fresh B mix solution was made, and 15 µL was added to the tube, which was then was heated to 37°C for 45 minutes, and then to 95°C for 10 minutes. Fresh C mix (15 µL) was added to the tube, heated (95°C for 10 minutes), followed by 35 cycles each of denaturation (95°C for 5 seconds), primer annealing (55°C for 30 seconds) and primer extension (72°C for 30 seconds), with a final incubation of 95°C for 2 minutes. Detection was carried out using the ArrayTube embedded with multiple oligonucleotide control spots which measure the efficiency of critical steps and a series of 14 specific ligation detection reaction (LDR) probes [43, 49] (Clondig, Jena, Germany) supplied in the PTS kit. Hybridization buffer (300 µL) was added to the ArrayTube and preheated to 50°C in the Thermo-Mixer. Amplified sample (10 µL) was added to the tube, incubated (50°C for 30 minutes at 400 rpm) and followed by a wash with 300 µL of blocking buffer (50°C for 5 minutes at 400 rpm). Blocking buffer was replaced with 300 µL of fresh blocking buffer, the temperature of the Thermo-Mixer was decreased to 30°C, and the tube was then incubated (10 minutes at 400 rpm). Fresh conjugate was prepared by adding 5 µL of conjugate solution with 495 µL of detection buffer. Blocking buffer was removed from the array tube, and 150 µL of conjugate was added to the tube and incubated (15 minutes at 30°C at 400 rpm). Conjugate was

removed, replaced with 600 µL of detection buffer, and the tube was incubated (30°C at 400 rpm for 2 minutes). Detection buffer was decanted, replaced with 600 µL of fresh detection buffer, and incubated (2 minutes at 30°C and 400 rpm). Finally, detection buffer was removed and 150 µL of staining solution was added to the array tube, and incubated (room temperature for 15 minutes). DNA hybridization results were analyzed after 15 minutes on the ATR-03 (Clondig, Jena Germany) using Check-Points software version 7.1.

xMAP *Salmonella* serotyping assay. For DNA extraction, isolates were inoculated to blood agar base slants and incubated (37°C overnight). An inoculating needle was used to transfer a small amount of growth into a 0.2 µL PCR tube containing 25 µL of Instagene Matrix (Bio-Rad, Hercules, California) and vortexed for 30 seconds. Tubes were centrifuged (2000 x g, 10 sec), placed in a C1000 Thermocycler (Bio-Rad, Hercules, California) and heated at 56°C for 10 minutes and 100°C for 5 minutes. Tubes were removed, 75 µL of nuclease-free water was added to each sample, tubes were vortexed for 30 seconds and then centrifuged (2000 x g, 5 minutes). The supernatant was carefully removed from the pellet and used as the DNA template for the PCR. For amplification, custom PCR primers were synthesized (Integrated DNA Technologies, Coralville, Iowa) according to the sequences described [39, 40]. Three PCR master mixes containing *Taq* polymerase, primers, dinucleotide triphosphates, magnesium chloride and water were prepared for each of the three typing assays: the O antigen assay; the H antigen assay; and the alternative targets (AT) assay. To provide for uniformity among the samples, each master mix was prepared in a 2 mL tube. Each reaction consisted of 12.5 µL of

Qiagen HotStar Taq Mastermix, (Qiagen, Valencia, California) 2.5 µL of appropriate primer pool, and 8 µL of nuclease-free water. 23 µL of each master mix was pipetted into 0.2 µL reaction tubes to which was added 2 µL of sample DNA, for a total of 25 µL per tube. Two µL of nuclease-free water served as the no-template control; 2 µL of DNA from a known isolate of *Salmonella* serotype Enteritidis was used as a positive control. Tubes were vortexed for 15 seconds and then briefly centrifuged (2000 x g) to remove any fluid from the tops of the tubes. Tubes were placed in the BioRad C1000 Thermocycler and the assay was run under the following parameters: an initial cycle of heating (95°C for 15 minutes) to activate the *Taq* polymerase, followed by 30 cycles each of denaturation (94°C for 30 seconds), primer annealing (48°C for 90 seconds), and primer extension (72°C for 90 seconds) and a final incubation at 72°C for 10 minutes. For the hybridization reaction, oligonucleotide probes, specific to a particular *Salmonella* marker, were synthesized and covalently linked to polystyrene microspheres each containing a unique fluorescent spectral address (Radix, Georgetown, Texas), as described [39, 40]. The bead mixes were stored at 4°C in the dark, at a concentration of 2.5 x10^6 microspheres per mL. The individual beads were combined to make up the three serotyping assays by adding equal volumes of each bead into an amber microcentrifuge tube (Diagger, Vernon Hills, IL). Five bead sets were combined to make the O antigen assay: Group B; Group C1; Group C2, Group D and Group E. The H antigen assay contained 36 bead sets: a; b; c; d; e,h; i; k; r; y; z; z6; z10; z29; and z35; 5 H complex antigens: G; EN; 1; L; and Z4; and 16 single factor antigens: 2; 5; 6; 7; f; m(g,m); m(m,t); p; s; t(m,t); v; x; z15; z24; ; z28; and z51. The AT assay was comprised of 3 different bead sets: one probe targeting the *sdf* gene, which is unique to *Salmonella* serovar

Enteritidis; a probe that targeted part of the *flj*B gene to identify diphasic *Salmonella*; and a probe that detects sequences encoding of the Vi antigen, *via*B, in *Salmonella* serovar Typhi.

Hybridization buffer was prepared fresh each day by diluting each of the bead sets into 1.5X TMAC buffer (tetramethylammonium chloride, Sigma-Aldrich, St. Louis, MO) to yield 2,500 microspheres of each antigen per mL. For each assay, 33 µL of hybridization buffer was added to 5 µL of PCR product and 12 µL of TE buffer (Tris-EDTA, pH 8.0, Sigma-Aldrich, St. Louis, MO) in an unskirted, 96-well low profile PCR plate (Bio-Rad, Hercules, CA). The products were heated to 94°C for 5 minutes, and then held at 52°C for 30 minutes for DNA denaturation. Fresh detection buffer was made by diluting SAPE (Streptavidin, R-Phycoerythrin conjugate, Invitrogen, Bethesda, MD) in 1X TMAC to a final dilution of 4 µg/mL. Detection buffer (75 µL) was added to the hybridization mix, and the samples were moved to a brass plate that was pre-heated to 52°C, and incubated (52°C for 10 minutes), in the Bio-Plex 200 Suspension Array instrument (Bio-Rad, Hercules, CA). Samples were analyzed for the mean fluorescence intensity (MFI) of each bead set and evaluated with comparison to the background fluorescence. In general, a positive signal was observed to be greater or equal to 1000; however, there was some variance in individual probes.

Data interpretation. Results of all three methods were evaluated and placed into one of three possible categories: correct, inconclusive, or incorrect. A distinction was made between results that were considered to be inconclusive versus results that were incorrect.

In the laboratory setting, additional testing will be required prior to reporting of results to the customer.

For classical serotyping and the xMAP *Salmonella* serotyping assay, results were considered correct when both the O and H antigens were identified completely in accordance with the Kauffmann-White scheme and matched the expected results. Results were considered incomplete when either the O, H1 or H2 antigens, or any combination of those antigens were not detected. Results were considered incorrect when the O, H1 or H2 antigens or any combination of those antigens did not match expected results and differed from the other two tests.

For the PremiTest assay, results were scored as correct when the named serovar matched the expected results based on classical typing. Results were regarded as inconclusive when two or more serovar names or codes were given, or when the results were listed as "DNA not OK" or "*Salmonella* species". Results were considered to be incorrect if the identified serovar did not match the expected results and differed from the other two tests.

CHAPTER 4. RESULTS

Evaluation of classical *Salmonella* serotyping. A total of 233 isolates of *Salmonella* were initially screened (Tables 1 and 2). Of these, 212 isolates (91%) had complete and correct serovar results with *Salmonella* O and H antisera (Table 1). Nineteen isolates (19/233, 8%) were untypeable by classical serology due to a rough O reaction, poor or non-motile H antigen, or because the isolate contained multiple serovars, indicating mixed cultures (Table 2). NVSL does not routinely provide serovar information on samples that are received that do not contain a single isolated colony of *Salmonella*. Of those which could be typed, an overall error rate of less than 1 % (2/233 isolates) was achieved with classical typing. Two isolates were incorrectly serotyped. One sample characterized as *Salmonella* serovar Thompson via the PTS and xMAP *Salmonella* assay had been incorrectly identified as *Salmonella* serovar Choleraesuis var. Kunzendorf via classical typing. Classic typing also designated one sample as *Salmonella* serovar Typhimurium, however, both molecular assays indicated that the isolate was *Salmonella* serovar Saintpaul. Ninety-eight isolates were identified that represented the top ten serovars that were observed at the NVSL over the last decade (Table 3).

Evaluation of the PremiTest *Salmonella* assay. A total of 233 isolates of *Salmonella* were tested on the PremiTest assay (Tables 1 and 2). Of these, 150 isolates (64%) had complete and correct serovar results. Sixty-four isolates (28%) did not have definitive results; 16 listed dual serovars, 33 isolates had results of a unique genovar code, 9 isolates were listed as *Salmonella* species, and 6 isolates showed assay failures. An overall error rate of 8 % (19/233 isolates) was obtained via testing with the PTS. Of the

19 isolates that were incorrectly typed, nine were not covered in the assay and were not expected to produce a serovar name [9]. *Salmonella* serovar Abetetuba was incorrectly identified as *Salmonella* serovar Carrau. *Salmonella* serovar Istanbul was identified as *Salmonella* serovar Hadar. *Salmonella* serovar Krefeld was identified as *Salmonella* serovar Gloucester. Two isolates of *Salmonella* serovar Johannesburg were both identified as *Salmonella* serovar Urbana, and four different serovars were all incorrectly identified as *Salmonella* serovar Senftenberg: *Salmonella* serovar Uganda, *Salmonella* serovar Apapa, *Salmonella* serovar Inverness, and *Salmonella* serovar Putten. Ten additional isolates that were identified incorrectly on the PremiTest were expected to be correctly typed on the basis of assay coverage [9]. These included two isolates of *Salmonella* serovar Anatum which were incorrectly typed as *Salmonella* serovar Newport or Oranienburg and *Salmonella* serovar Brandenburg. One isolate each of *Salmonella* serovar Kentucky and *Salmonella* serovar Give were identified as *Salmonella* serovar Manchester. *Salmonella* serovar Dublin was identified as *Salmonella* serovar Manhattan, *Salmonella* serovar Orion was identified as *Salmonella* serovar Livingstone, *Salmonella* serovar Senftenberg was identified as *Salmonella* serovar Newport, *Salmonella* serovar was identified as *Salmonella* serovar Schwarzengrund or Grumpensis, and two isolates of *Salmonella* serovar Schwarzengrund were identified incorrectly. The first was identified as *Salmonella* serovar Poona and the second was identified as *Salmonella* serovar Altona. When only the 98 isolates that represent the top ten serovars observed at the NVSL over the last decade are considered (Table 3), the PTS correctly identified 87 (89%), 7 isolates were inconclusive (7%) and 4 isolates (4%) were incorrectly identified as a different serovar.

Evaluation of the xMAP *Salmonella* serotyping assay. A total of 233 isolates of *Salmonella* were analyzed on the xMAP assay (Tables 1 and 2). Of these, 181 isolates (78%) had complete and correct serovar results with *Salmonella* O and H microspheres. Fifty isolates (21%) did not have complete serovar information given on the xMAP assay; 11 isolates did not have an O antigen determined, 24 isolates were missing an H antigen, and 15 isolates were missing both O and H antigens. An overall error rate of less than 1 % (2/233 isolates) was obtained via testing with the xMAP assay. Two isolates were typed incorrectly on the xMAP assay. One isolate of *Salmonella* serovar Dublin was incompletely typed on the xMAP. *Salmonella* serovar Dublin is an O group D, and is a monophasic culture with g,p as the H1 antigen: (D:g,p:-). The antigenic formula on the xMAP for this isolate was E:L complex,-:1,5. The second isolate that was incorrectly typed was *Salmonella* serovar Fresno, which is also a monophasic culture. The O group is D, and the H1 antigen is z38, which is not an available bead on the assay: (D:z38:-). On the xMAP assay, the isolate showed O group D and H1 as the Z4 complex. When considering only the 98 isolates that represent the top ten serovars observed at the NVSL over the last decade (Table 3), the xMAP assay correctly identified 86 (88%), 12 isolates (12%) of *Salmonella* serovar Senftenberg were not completely typed. There were no isolates incorrectly typed.

CHAPTER 5. DISCUSSION

Classical serotyping has been an invaluable tool for characterizing *Salmonella* for surveillance and epidemiology; however, it does have its limitations. Obtaining serovar identification is dependent upon the phenotypic expression of surface antigens. When these antigens are incompletely expressed, definitive serovar identification cannot be achieved. DNA based assays such as the PTS and the xMAP are able to overcome these obstacles by identifying the genes that are responsible for encoding these antigens.

This study highlighted a few of the weaknesses of classical serotyping. One of the problems that can be encountered is the variability in the expression of the flagellar antigens. One serovar was incorrectly identified as *Salmonella* serovar Choleraesuis var. Kunzendorf. It has been suggested that this serovar does not always exhibit the first phase H (H1) antigen [3]. Because of this known aberration, if the H1 antigen is absent, but the remaining antigens and biochemical reactions are correct, and the isolate is of swine origin, the isolate is designated as *Salmonella* serovar Choleraesuis var. Kunzendorf. This isolate met the above criteria: it was of swine origin, the O antigen and the second phase H antigen, and the biochemical profile matched that of *Salmonella* serovar Choleraesuis var. Kunzendorf. However, the H1 antigen was absent. Further analysis by molecular typing on both the PTS and the xMAP assays showed that the antigenic composition of the isolate matched that of *Salmonella* serovar Thompson. Serological testing was repeated and the results were consistent with the original results. It is believed that the molecular assays were correct and that the unexpressed first phase H antigen was a “k” as opposed to that of *Salmonella* serovar Choleraesuis, which, when expressed,

would be “c.” NVSL has since changed its policy and will no longer assign a serovar name to an isolate that is missing components of the antigenic structure when testing by classical serotyping. A second isolate was incorrectly typed as *Salmonella* serovar Typhimurium. Molecular analysis via the PTS and the xMAP assays indicated that the sample was *Salmonella* serovar Saintpaul. Serological testing was repeated and also confirmed that the isolate was *Salmonella* serovar Saintpaul. An overall error rate of less than 1 % (2/233 isolates) was achieved with classical typing. Nineteen isolates (8%) were considered to be untypeable by classical serotyping due to rough O antigens or lack of expression of one or both H antigens. Analysis by the two molecular assays gave new insight into three of these isolates. One of the two samples that were identified as 4,(5),12:r:- through classical serotyping had the same results on the xMAP assay, and the marker for the *flj*B gene was also negative, indicating that the gene for the second phase H antigen was incomplete or missing. The PTS identified this isolate as *Salmonella* serovar Heidelberg. The PTS does not have a name built into the database for this serovar; however, the results given are not entirely inconsistent with the results obtained from the other two tests. One of the three samples that were classically serotyped as 4,(5),12:i:- had the exact same results on the PTS and the xMAP assays. This serovar, although not named, is accounted for on the PTS. The second of the three showed the same results on classical serotyping and the xMAP, and the marker for the *flj*B gene was also negative, however, the PTS identified the isolate as *Salmonella* serovar Typhimurium. Again, this identification is not completely inconsistent with the other two tests. Results for the remaining 16 isolates that were considered incomplete with classical serotyping were consistent with the serovars that were identified with molecular typing.

Of the top ten serotypes received at the NVSL, the PTS was able to successfully identify 89% of the isolates. Seven percent were inconclusive and four percent were incorrect. However, when looking at a wider variety of isolates, the PTS was able to successfully type 64% of the isolates. Sixty-four total isolates (27%) had inconclusive results on the PTS. Of these, there were five different serovars that had multiple isolates tested which gave unique genovar codes for at least two isolates in the set. Two isolates of *Salmonella* serovar Alachua were given the genovar 4212. Three isolates of *Salmonella* serovar Give were tested. Two isolates gave the unique genovar code of 14399, and one isolate was incorrectly named *Salmonella* serovar Manchester. Three isolates of *Salmonella* serovar Uganda were tested. Two of the three gave the unique genovar code 13487, and one isolate was given an incorrect name, *Salmonella* serovar Senftenberg. Of six isolates of *Salmonella* serovar Worthington, three isolates were given the correct serovar name, and three isolates gave the unique genovar code of 14369. Eleven isolates of *Salmonella* serovar Kentucky, along with two isolates that were incompletely typed by classical serology as rough O:i:z6 and 8,(20):-:z6 that were confirmed as *Salmonella* serovar Kentucky on the xMAP assay were tested. Four isolates were correctly identified, six isolates were given the unique genovar of 10299 and two other isolates were given the unique genovar of 10283. One isolate had results that gave three different possibilities; *Salmonella* serovar Manchester, genovar 10299 and genovar 10283. Additional testing on these serovars to ensure consistent results could allow the manufacturer to add them to the database. If these codes had been included in the database, the PTS would have identified 17 additional isolates bringing the total of complete results to 167 (72%). There was another interesting observation with the PTS assay. As stated previously, when

multiple isolates of the same serovar were chosen, great care was taken to select those that represented unique strains among the same serovar by picking isolates that were from different species, different time periods, and/or from different locations. The incomplete or incorrect results were not evenly distributed among all of the serotypes, rather, the PTS was able to completely identify all isolates of some serovars, and able to identify only a few isolates of other serovars. There were 13 different serovars that had multiple isolates tested that the PTS was not able to identify. Three of those, *Salmonella* serovars Give, Kentucky and Worthington were previously discussed, as the PTS gave consistent unique genovar codes to isolates in those groups. Ten additional serovar sets also proved problematic. Six isolates of *Salmonella* serovar Anatum were tested, four isolates were correctly identified, and two isolates were incorrectly named *Salmonella* serovar Newport or Orainienburg and *Salmonella* serovar Brandenburg. Five isolates of *Salmonella* serovar Braenderup were tested; three were correctly identified, one isolate was given a unique genovar code and one isolate was reported as *Salmonell*a suspected. Five isolates of *Salmonella* serovar Cerro were tested, with three correctly named, one reported as 4,(5),12:i:- or *Salmonella* serovar Cerro, and one reported as *Salmonella* serovar Senftenberg or *Salmonella* serovar Cerro. Four isolates of *Salmonella* serovar Choleraesuis were tested. Two isolates were reported as *Salmonella* serovar Choleraesuis or *Salmonella* serovar Paratyphi, one isolate was reported as “DNA not OK”, and one isolate was reported as serotype not determined. Seven isolates of *Salmonella* serovar Dublin were tested. Four isolates were correctly identified, one isolate was given a unique genovar code, one isolate was identified as *Salmonella* serovar Banana or *Salmonella* serovar Dublin and one isolate was incorrectly named *Salmonella* serovar

Manhattan. Four isolates of *Salmonella* serovar Meleagridis were tested. Two isolates were correctly identified, one isolate was given a unique genovar code, and one isolate was incorrectly identified as *Salmonella* serovar Schwarzengrund or *Salmonella* serovar Grumpensis. Eight isolates of *Salmonella* serovar Muenster were tested. Six isolates were identified as *Salmonella* serovar Munster or *Salmonella* serovar Reading, one isolate was reported as "DNA not OK" and one isolate was reported as *Salmonella* species. Three isolates of *Salmonella* serovar Orion were tested. One isolate was incorrectly identified as *Salmonella* serovar Livingstone, and two isolates were reported as DNA not OK. Five isolates of *Salmonella* serovar Schwarzengrund were tested. Three isolates were identified as *Salmonella* serovar Schwarzengrund or *Salmonella* serovar Grumpensis, and two isolates were incorrectly named. One isolate was identified as *Salmonella* serovar Poona and the other was identified as *Salmonella* serovar Altona. Two isolates of *Salmonella* serovar Johannesburg, which were not on the list of serovars that were covered on the PTS assay, were incorrectly identified as *Salmonella* serovar Urbana. Incorrect results were given for 19 isolates on the PTS. Of these, nine isolates were not expected to give a serovar result due to non-coverage on the assay. Ten more isolates were expected to be correctly typed.

The European-based PTS offers a new multiplex format for rapid identification of *Salmonella* serovars that is based on genetic markers and not phenotypic expression. Test time required from recovery of a single colony until the isolate is identified in approximately eight hours, which has significant advantages over the three or more days required for classical serotyping. The PTS equipment has a small footprint and could

easily be incorporated into any laboratory that does serotyping. As more isolates are tested, information could be added to the PTS database and released as simple software updates. There are some issues that need to be addressed by the manufacturer, however. The current price of approximately $44 per isolate will significantly limit the number of laboratories that will be able to afford the technology. Although the PTS did well at identifying 89% of the top ten serotypes, it had a four percent error rate on those isolates as well. When all 233 isolates representing more than 52 serovars were considered, the PTS was able to identify just 64% of the isolates correctly, and it had an error rate of nine percent. Based on these results, a reference laboratory that receives a wide variety of isolates may find the specificity of the assay to be problematic. Finally, there were a few issues with the timing of the final staining step and the subsequent analysis by the ATR. The manufacturer indicates that the array tube must be read 15 minutes after staining. This presented a logistical problem if several isolates were tested at the same time, which is one of the advantages of such an assay. Immediately before the array tube could be read, a unique 11 digit code had to be entered into the software for each of the three isolates in the tube. If 21 isolates (7 array tubes) were being tested at a time, more than 20 minutes had passed before the last array tube could be read. Changing the software to allow for the entering of sample information prior to running the assay would resolve this issue. There was also significant variability in the timing of the analysis that could not be traced to any specific serovar or other circumstance. There would be occasions where at 15 minutes, the stain in the array tube was not developed and results could not be determined. Re-analyzing after 25 minutes would then yield a serovar result. On other occasions, the array tube would be over-stained at 15 minutes, and results would not be

obtained. Following this observation, tubes were analyzed at both 15 and 25 minutes for the remainder of the project. This did resolve some issues, however, there were times when the 15 minute read would provide one serovar name, and the 25 minute read would provide a different serovar name. It was decided that, in this project, the first serovar name that was given would be accepted as the serovar name upon which results would be based, as this was in accordance with the manufacturer's directions. The assay, at tested conditions, is a rapid screening tool that provides valuable information about the samples being tested. Serovar identification should be confirmed using the data provided by the PTS either in-house or through a diagnostic reference laboratory.

When considering the top ten serovars received by the NVSL, the xMAP assay was able to successfully type 88% of the isolates. Twelve percent were partially typed, and there were no incorrect results. The xMAP was able to completely type 78% of all 233 isolates tested and 21% of all isolates had partial results. Two isolates, *Salmonella* serovar Dublin and *Salmonella* serovar Fresno gave unexpected results. The expected results of *Salmonella* serovar Dublin were D:g,p:-, however the actual results on the xMAP were E:L complex,-:1,5. The PTS and classical serotyping both indicated that the isolate was *Salmonella* serovar Dublin. Additional testing was not an option due to loss of viability of the isolate. The possibility of cross contamination was considered, but there were no other isolates that were tested at that time with antigens similar to the results obtained. The expected results for *Salmonella* serovar Fresno on the xMAP are D:-:-, as the z38 antigen is not included in the assay. The actual results that were observed on the xMAP were D:Zcomplex,-:-. This isolate was not included in the PTS database, and it was

identified with a unique genovar code. Retesting with classical typing agreed with the original results. Five isolates, *Salmonella* serovar Abetetuba, *Salmonella* serovar Barranquilla, *Salmonella* serovar Cubana, *Salmonella* serovar Mississippi and *Salmonella* serovar Rubislaw, were incompletely typed on the xMAP assay, due to non-coverage of their O antigens. Updates to the assay have been made since testing, and these isolates can now be completely typed on the xMAP. Based on the O and H beads that were included in the assay at the time of testing, 181 of the 233 isolates were predicted to give complete serovar identification (Table 4). The xMAP had complete coverage of 90% of these isolates. One isolate of *Salmonella* serovar Dublin that was expected to be correctly identified gave incorrect results. All 12 isolates of *Salmonella* serovar Senftenberg were incompletely typed. The expected results for this were E:g,s,t:-, however, the actual results for all isolates were E:g,s:-. The xMAP assay does have a bead representing single factor “t”, but, this antigen is known to have 3 lineages, and the “t” bead in the assay does not currently represent all lineages [39, 50]. *Salmonella* serovar Taksony had expected results of E:i:z6 on the xMAP assay, however, the actual results were -:i:z6. Additional isolates were not tested, so, it is not known if this is a problem with the serovar or if it was related to the actual isolate tested. Classical serotyping was repeated and the results were consistent with original testing. *Salmonella* serovar Muenster did not produce consistent results on the xMAP assay. The expected results were E:e,h:1,5. The actual results from four isolates was E:e,h:1,-. The other four isolates were identified as E:e,h:1,5, although results from the single factor “5” bead were weaker than other serovars expressing single factor “5”. *Salmonella* serovar Munster is known to variably express the antigen for single factor five, which is not considered to be of

epidemiological significance [50]. In total, 11 isolates were missing O antigen results, 24 isolates were missing an H antigen result, and 15 isolates were missing both an O and H antigen result due to lack of coverage of the antigens on the assay.

The xMAP offers a rapid molecular alternative to classical serotyping in a 96-well multiplex format. Typing time is approximately five hours from a single colony. The xMAP assay considers the O and H antigens on a molecular level, and is not constrained to the vagaries of phenotypic expression that occur with classical serotyping. The assay is relatively simple to perform and the results are easily interpreted. The price is approximately $10-$15 per isolate, which will allow many laboratories to utilize it. Finally, the biggest advantage of the xMAP assay is the ability to obtain partial serovar information. Because the results are based on the O and H antigens, information obtained can be compared against the Kauffmann-White scheme and the isolate tested only for the remaining antigens. Incorporating the results from the xMAP into classical serotyping shortens the necessary time to type, decreases the amount of antisera that is required to test and potentially eliminates the need to perform phase inversion on an isolate. Although the xMAP was not able to completely identify all 233 isolates that were tested, with the exception of the one isolate of *Salmonella* serovar Dublin which had incorrect results for both the O and H antigens, the assay provided correct information for at least part of each isolate tested. Future development of beads targeting additional O and H antigens can easily be incorporated into the assay. The issues of the xMAP not detecting some of the single factors that appear to be present in the assay have been previously noted by McQuiston and Fitzgerald. Future releases may contain beads that better

represent these isolates. The xMAP *Salmonella* assay provides a relatively inexpensive alternative to classical serotyping for many laboratories that are testing the most common serotypes. Time to test from an isolated colony is about five hours as opposed to the several days necessary for classical serotyping. If complete information for a serovar is not obtained, testing needs to be performed for only the missing antigens. The error rate for the xMAP is less than one percent, which is comparable to that observed with classical serotyping. As developed, the xMAP assay can be used as a complement to classical typing; however, it will not replace the need for additional antisera, as there are many isolates of significance to both human and animal health which do not yet have complete serovar coverage.

Table 1. Results of serovars tested by method

		Kauffmann-White			PremiTest *Salmonella*			xMAP *Salmonella*		
Serovar	Number of samples	Correct serovar	Inconclusive serovar	Wrong serovar	Correct serovar	Inconclusive serovar	Wrong serovar	Correct serovar	Inconclusive serovar	Wrong serovar
Abetetuba	1	1	0	0	0	0	1	0	1[a]	0
Agona	9	9	0	0	9	0	0	9	0	0
Alabama	1	1	0	0	0	1	0	1	0	0
Alachua	2	2	0	0	0	2	0	0	2	0
Albert	1	1	0	0	0	1	0	1	0	0
Altona	2	2	0	0	2	0	0	2	0	0
Amager	1	1	0	0	0	1	0	1	0	0
Anatum	6	6	0	0	4	0	2	6	0	0
Apapa	1	1	0	0	0	0	1	0	1	0
Barranquila	1	1	0	0	0	1	0	0	1[a]	0
Berta	1	1	0	0	1	0	0	1	0	0
Braenderup	5	5	0	0	3	2	0	5	0	0
Bredeney	1	1	0	0	1	0	0	1	0	0
Cerro	5	5	0	0	3	2	0	0	5	0
Cholerasuis	4	4	0	0	0	4	0	4	0	0
Cubana	1	1	0	0	1	0	0	0	1[a]	0
Derby	5	5	0	0	5	0	0	5	0	0
Dublin	10	10	0	0	7	2	1	9	0	1
Enteritidis	13	13	0	0	13	0	0	13	0	0
Fresno	1	1	0	0	0	1	0	0	0	1
Gallinarum	2	2	0	0	2	0	0	2	0	0
Gaminara	1	1	0	0	0	1	0	0	1	0
Give	3	3	0	0	0	2	1	3	0	0
Hadar	10	10	0	0	9	1	0	10	0	0
Heidelberg	11	11	0	0	11	0	0	11	0	0
Infantis	5	5	0	0	5	0	0	5	0	0
Inverness	1	1	0	0	0	0	1	0	1	0

[a]Additional O beads were not available for complete serotyping of these isolates at the time of the study,

Table 1. Continued

Istanbul	1	1	0	0	0	0	1	1	0	0
Johannesburg	2	2	0	0	0	0	2	0	2	0
Kentucky	11	11	0	0	4	6	1	11	0	0
Kiambu	1	1	0	0	0	1	0	1	0	0
Krefeld	1	1	0	0	0	0	1	0	1	0
London	2	2	0	0	2	0	0	2	0	0
Mbandaka	5	5	0	0	5	0	0	5	0	0
Meleagridis	4	4	0	0	2	1	1	0	4	0
Mississippi	1	1	0	0	0	1	0	0	1[a]	0
Montevideo	10	10	0	0	10	0	0	10	0	0
Muenchen	1	1	0	0	1	0	0	1	0	0
Munster	8	8	0	0	0	8	0	4	4	0
Newport	10	10	0	0	9	1	0	10	0	0
Norwich	1	1	0	0	0	1	0	1	0	0
Orion	3	3	0	0	0	2	1	3	0	0
Ouakam	1	1	0	0	1	0	0	1	0	0
Pullorum	5	5	0	0	4	1	0	5	0	0
Putten	1	1	0	0	0	0	1	0	1	0
Rubislaw	1	1	0	0	0	1	0	0	1[a]	0
Saintpaul	1	0	0	1	1	0	0	1	0	0
Schwarzengrund	5	5	0	0	0	3	2	5	0	0
Senftenberg	12	12	0	0	11	0	1	0	12	0
Soerenga	1	1	0	0	0	1	0	0	1	0
Taksony	1	1	0	0	0	1	0	0	1	0
Thompson	1	0	0	1	1	0	0	1	0	0
Typhimurium	11	11	0	0	11	0	0	11	0	0
Uganda	3	3	0	0	0	2	1	0	3	0
Worthington	6	6	0	0	3	3	0	0	6	0
TOTALS	214	212 (99%)	0 (0%)	2 (1%)	141 (66%)	54 (25%)	19 (9%)	162 (76%)	50 (23%)	2 (1%)

Table 2. Results of PremiTest Salmonella and xMAP Salmonella on classically untypeable strains

		PremiTest *Salmonella*		xMAP *Salmonella*	
Serovar	Number of Samples	Correct serovar	Inconclusive serovar	Correct serovar	Inconclusive serovar
3,15:e,h:-	1	0	1	1	0
4,(5),12:r:-	2	1	1	2	0
4,5,12:i:-	3	2	1	3	0
4,5,12:Non-motile	1	0	1	1	0
8,(20):-:z6	1	0	1	1	0
9,12:Non-motile	3	3	0	3	0
Kentucky & Heidelberg	1	0	1[a]	1	0
Rough O:f,g,s:-	2	2	0	2	0
Rough O:g,p	1	0	1	1	0
Rough O:i:1,2	1	0	1	1	0
Rough O:i:z6	1	0	1	1	0
Rough O:k:1,5	1	1	0	1	0
Rough O:r:1,2	1	0	1	1	0
Total	19	9 (47%)	10 (53%)	19(100%)	0

[a]Submitted culture contained two serovars

Table 3. Results by method of the top ten serovars received at the National Veterinary Services Laboratories

		Kauffmann-White			Premitest *Salmonella*			xMAP *Salmonella*		
Serovar	Number of Samples	Correct serovar	Inconclusive serovar	Wrong serovar	Correct serovar	Inconclusive serovar	Wrong serovar	Correct serovar	Inconclusive serovar	Wrong serovar
Typhimurium	11	11	0	0	11	0	0	11	0	0
Heidelberg	11	11	0	0	11	0	0	11	0	0
Newport	10	10	0	0	9	1	0	10	0	0
Kentucky	11	11	0	0	4	6	1	11	0	0
Senftenberg	12	12	0	0	11	0	1	0	12	0
Enteritidis	13	13	0	0	13	0	0	13	0	0
Montevideo	10	10	0	0	10	0	0	10	0	0
Derby	5	5	0	0	5	0	0	5	0	0
Agona	9	9	0	0	9	0	0	9	0	0
Anatum	6	6	0	0	4	0	2	6	0	0
TOTAL	98	98 (100%)	0 (0%)	0 (0%)	87 (89%)	7 (7%)	4 (4%)	86 (88%)	12 (12%)	0 (0%)

Table 4. Predicted versus actual identification of serovars tested by molecular assay

		PremiTest *Salmonella*		xMAP *Salmonella*	
Serovar	Number of Samples	Predicted correct	Actual correct	Predicted correct	Actual correct
Abetetuba	1	0	0	0	0
Agona	9	9	9	9	9
Alabama	1	0	0	1	1
Alachua	2	0	0	0	0
Albert	1	0	0	1	1
Altona	2	2	2	2	2
Amager	1	0	0	1	1
Anatum	6	6	4	6	6
Apapa	1	0	0	0	0
Barranquila	1	0	0	0	0
Berta	1	1	1	1	1
Braenderup	5	5	3	5	5
Bredeney	1	1	1	1	1
Cerro	5	5	3	0	0
Cholerasuis var Kunzendorf	4	4	0	4	4
Cubana	1	1	1	0	0
Derby	5	5	5	5	5
Dublin	10	10	7	10	9
Enteritidis	13	13	13	13	13
Fresno	1	0	0	0	0
Gallinarum	2	2	2	2	2
Gaminara	1	0	0	1	1
Give	3	3	0	3	3
Hadar	10	10	9	10	10
Heidelberg	11	11	11	11	11
Infantis	5	5	5	5	5
Inverness	1	0	0	0	0
Istanbul	1	0	0	1	1
Johannesburg	2	0	0	0	0
Kentucky	11	11	4	11	11
Kiambu	1	0	0	1	1
Krefeld	1	0	0	0	0
London	2	2	2	2	2
Mbandaka	5	5	5	5	5
Meleagridis	4	4	2	0	0
Mississippi	1	0	0	0	0
Montevideo	10	10	10	10	10
Muenchen	1	1	1	1	1
Munster	8	8	0	8	4
Newport	10	10	9	10	10
Norwich	1	0	0	1	1
Orion	3	3	0	3	3
Ouakam	1	1	1	1	1
Pullorum	5	5	4	5	5
Putten	1	0	0	0	0
Rubislaw	1	0	0	0	0
Saintpaul	1	1	1	1	1
Schwarzengrund	5	5	0	5	5
Senftenberg	12	12	11	12	0
Soerenga	1	0	0	0	0
Taksony	1	0	0	1	0
Thompson	1	1	1	1	1
Typhimurium	11	11	11	11	11
Uganda	3	0	0	0	0
Worthington	6	6	3	0	0
TOTALS	214	189	141 (75%)	181	163 (90%)

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